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Abstracts

Eosinophilopoiesis*

To be or not to be (an eosinophil)? That is the question: transcriptional mechanisms regulating eosinophil genes and development

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The molecular controls for eosinophil development from multipotential myeloid progenitors, and the mechanisms by which eosinophil-specific genes are expressed and regulated during eosinophil terminal differentiation, are incompletely understood. Recent studies of avian Myb-ets transformed multipotent haematopoietic progenitors suggest that eosinophil development proceeds via a common neutrophil/macrophage/eosinophil myeloid precursor that is dependent on PU.1 expression, with the decision to enter the eosinophil program specified by expression of C/EBPs (C/EBPα and/or β) in the context of GATA-1 (1). Impaired eosinophil development occurs in both C/EBPα and C/EBPε deficient mice (2,3), but PU.1 null mice have not been studied. To assess the activities of C/EBPs (α, β, ε) in eosinophil differentiation, we have analysed their roles in regulating the IL-5 receptor α subunit gene (IL-5Rα), expressed early in eosinophil commitment, and the major basic protein gene (MBP), expressed during terminal maturation, in human committed (AML14) promyelocyte (AML14.eos) and fully differentiated (AML14·3D10) eosinophil cell lines. Both IL-5Rα (P1) and MBP (P2) promoters (Fig. 1) possess functional C/EBP sites that bind C/EBPβ and C/EBPε in nuclear extracts of eosinophil-differentiated AML14·3D10 cells. In contrast, C/EBPα complexes were detected only in the parental AML14 and AML14.eos cell lines, but not in AML14·3D10. Both C/EBPε mRNA and binding activity was identified in all three cell lines, with AML14·3D10 ≫ AML14.eos > AML14· Human IL-5Rα promoter constructs were transactivated by C/EBPα, but not by C/EBP β, ε-long (32·2 kDa) or ε-short (14·3 kDa) isoforms. However, the MBP promoter was equally transactivated by C/EBPα or β, less so by ε-long, and not by C/EBPε-short. Of interest, the C/EBPε-short isoform inhibited C/EBPα or β transactivation of the IL-5Rα and MBP promoters. In C/EBPα null mice, expression of the IL-5Rα gene was reduced approx. five-fold, but was unaffected by the C/EBPε null mutation. MBP and eosinophil peroxidase (EPO) mRNAs were undetectable in C/EBPα null mice, but were only reduced by approx. 50% in C/EBPε null mice. These findings support the concept that different C/EBPs (α, β and ε) have distinct regulatory roles during the stages of commitment versus terminal differentiation. We reported previously that the MBP promoter also contains a functional GATA-binding site 7 bp down-stream of its C/EBP site, and is positively regulated by GATA-1, but negatively regulated by GATA-2 (4). We have now shown that C/EBPβ and GATA-1 can simultaneously bind their sites in the MBP promoter, interact physically, and synergize to produce a five-fold enhancement of activity above that of the individual factors in a heterologous cell line (5). The proximity of the GATA-1 and C/EBP sites suggested that these factors might physically interact, an observation confirmed by GST-fusion protein pull down experiments (Fig. 1) (5).

In addition to the regulation of the IL-5Rα subunit gene by C/EBPα, we had previously characterized the IL-5Rα P1 promoter and identified a 10 bp enhancer element, EOS1 (GTTGCCTAGG, bp −430 to −421), that bound nuclear factors of human eosinophilic cell lines (AML14, AML14.eos and AML14·3D10), and which was responsible for >90% of promoter activity in eosinophilic cell lines (6). Based on DNA sequence analysis, binding activities and methylation interference data, we have now identified the EOS1 enhancer (GTTGCCTAGGAGAC, bp −430 to −417) as an RFX transcription factor binding site, which binds RFX proteins as homo- and heterodimers or multimer complexes (Fig. 1). RFX proteins (RFX1-5 and RFXAP), a multigene family of transcription factors, bind to x-box elements critical for regulation of all MHC class II genes including HLA-DR, -DP and -DQ isotypes, the murine ribosomal rpL30α gene, and enhanced factor C (EF-C) sites in the promoters of viruses that include hepatitis B (HBV) and polyoma (Py). The EOS1 enhancer is essentially identical to the HBV and Py RFX binding sites, with a two nucleotide difference at the 3′-end. Gel shift competition analyses showed that formation of the EOS1 complexes with nuclear factors of AML14·3D10 cells was completely inhibited by the EF-C sites of Py and HBV, murine rpL30α, and HLA-DRA x-box site, partially by HLA-DPA, but not by oligonucleotides lacking palindromic (inverted repeat) sequences characteristic of viral RFX elements (the HLA-DQA x-box and a mutant of the IL-5Rα EOS1 site). The same protein-DNA complexes obtained using nuclear extracts of the AML14·3D10 cell line were obtained using the HLA-DRA x-box site as a probe instead of EOS1. Since transcription factors

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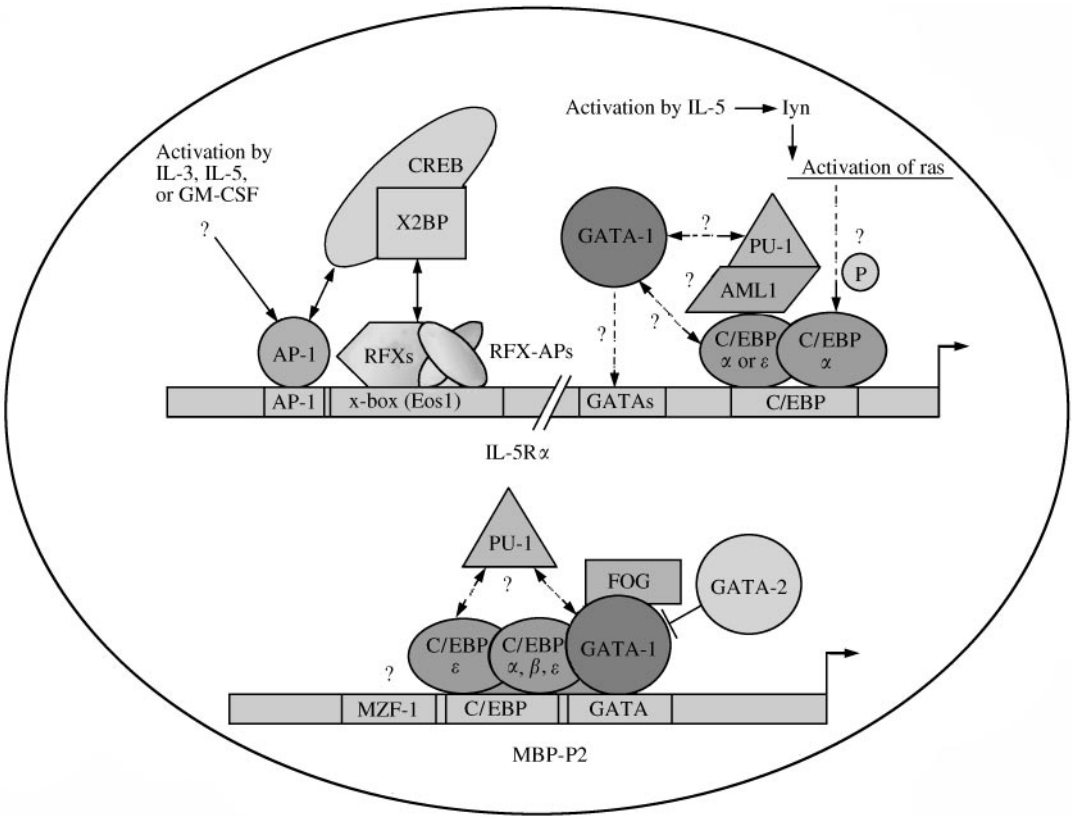

FIG. 1. Model for the transcriptional regulation of the genes encoding the IL-5 receptor α subunit (IL-5Rα) and granule major basic protein (MBP) in the eosinophil. The IL-5Rα-P1 and MBP-P2 promoters are shown schematically with dashed lines and/or question marks indicating potential interactions or binding sites based on studies of these and other myeloid gene promoters. For the IL-5Rα (P1) promoter, functionally active binding sites for members of the C/EBP and RFX transcription factor families have been characterized; for the MBP-P2 promoter, functionally active binding sites for the C/EBP and GATA transcription factor families are indicated. RFX proteins, which bind to an x-box (originally termed the Eos-1 site) may include RFX-1, -3, -5 and RFX-associated proteins (RFX-AP, RFX-B), and are known to interact with X2BP-CREB in the regulation of MHC Class II gene expression. In the IL-5Rα promoter, an inducibly active AP-1 binding site is located immediately upstream of the RFX-binding x-box; AP-1 also interacts with CREB. The IL-5Rα C/EBP site can be transactivated by C/EBPα and ε, but not C/EBPβ, whereas C/EBPα, β and ε all have activity either independently or are significantly increased in the context of GATA-1 and PU.1. Activated ras has been reported to phosphorylate and enhance the activity of C/EBPα for transactivation of the G-CSFR promoter and might function similarly in the eosinophil via the IL-5-induced lyn kinase pathway. The role for PU.1 in IL-5Rα and MBP regulation may be through AML1 and/or through interactions with GATA-1 and C/EBPα. For the MBP-P2 promoter, C/EBPα has been shown physically interact and synergize with GATA-1, whereas GATA-2 competes with GATA-1 and may act as a negative regulator. FOG (friend of GATA-1) acts as a positive co-factor for GATA-1 regulation of MBP in the eosinophil, in contrast to its role as a negative regulator in the erythroid lineage.

recognize and interact with DNA in distinct structural motifs, we modeled binding of the RFX complexes to the EOS1 element based on their methylation interference patterns using a cylindrical DNA helical projection. Over a length of two helical turns, all nuclear protein contacts indicated by methylation interference mapped to one side of the DNA helix, suggesting that the RFX protein complex binds the EOS1 element in the major groove, across the minor groove, and on only one side of the DNA helix. The model also reveals a diad symmetry in the binding site, consistent with the inverted repeat structure of RFX sites in viral promoters. RFX proteins, which contain both activation and repression domains, are expressed in cell-type and tissue-specific patterns. EOS1 binding complexes with RFX family members were detected in eosinophil (AML14, AML14·3D10, HL-60-C15) and other myeloid (HL-60, U937), lymphoid (B and T) and non-haematopoietic (HeLa) cell lines, but not in fibroblasts (NIH3T3) or COS-7 cells. Expression of RFX1, RFX3, RFX5 and RFXAP mRNAs was detected in both eosinophilic cell lines and purified peripheral blood eosinophils from a patient with the hypereosinophilic syndrome. In bone marrow-derived CD34+ stem cells, RFX5 and RFXAP were constitutively expressed, whereas RFX1 and RFX3

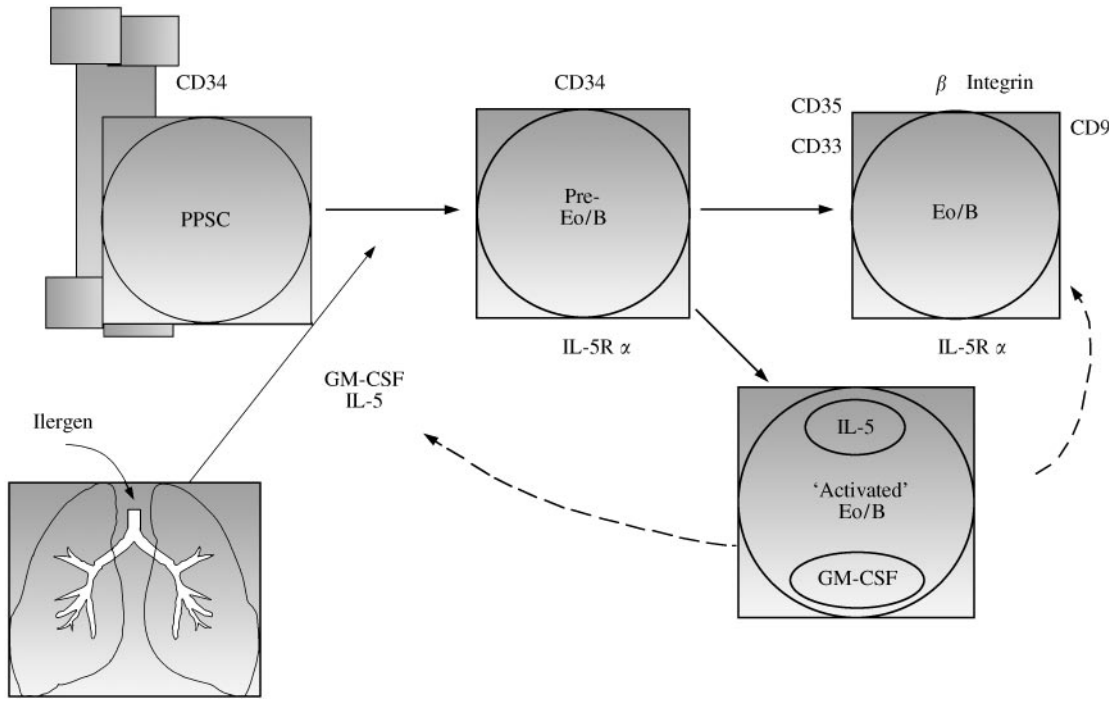

FIG. 2 Elicitation and activation of eosinophil–basophil lineage commitment after airway allergen challenge. Eo/B: eosinophil/basophil; GM-CSF: granulocyte–macrophage colony-stimulating factor; IL-5: interleukin 5; IL-5R″: high affinity IL-5 receptor″ subunit; PPSC: pluripotent stem cell.

expression was upregulated in response to IL-3 and/or IL-5 induced eosinophilic differentiation of the CD34+ cells. Our results indicate that RFX proteins are expressed in eosinophils, and bind the IL-5Rα EOS1 enhancer as homodimers, heterodimers or multimer complexes. These findings indicate that RFX proteins, in addition to C/EBPs, play a key role in the transcriptional regulation of IL-5Rα gene expression during both early eosinophil development and in the mature granulocyte.

Disruption of the PU.1 gene (PU.1−/−) severely impairs development of both lymphoid and myeloid lineages, resulting in either embryonic lethality by day 16–18 of gestation (7) or viable mice with no detectable monocytes, B cells and delayed/reduced neutrophil development (8). PU.1 gene disruption causes a severe reduction but not elimination of myeloid progenitors, which are still capable of responding to multilineage cytokines, but not to myeloid-specific cytokines. PU.1−/− progenitors can undergo only limited differentiation into neutrophils and monocytes. We have shown that human eosinophils express PU.1 during IL-5 induced eosinophilic development of CD34+ progenitors, but nothing was known of the effect of the PU.1 knockout on eosinophilopoiesis. We have used RT-PCR to analyse expression of the IL-5Rα gene, and the lineage-specific EPO and MBP genes in the PU.1 null mice. Our results show that IL-5Rα, EPO and MBP mRNAs are not expressed in fetal livers of PU.1−/− embryos. In contrast, IL-5Rα expression was reduced by approx. three to four fold in day 9 spleens of viable PU.1−/− mice, whereas MBP and EPO expression levels were identical to PU.1+/+ and PU.1+/− mice. Histochemistry showed eosinophils in the bone marrow and spleens of PU.1+/+ and PU.1+/−, but not d16.5 or viable PU.1−/− mice. These data indicate that eosinophil gene expression is still detectable in the absence of eosinophil terminal differentiation in both the embryonic lethal (d16.5) and viable PU.1 knockouts, supporting the idea that PU.1 is not essential for specification of eosinophil precursors, but controls their proliferation and terminal differentiation by regulating expression of growth factor receptor and other lineage-specific genes (Fig. 1).

In summary, these findings support the concept that the decision 'to be or not to be (an eosinophil)', is regulated by coordinate actions of C/EBPs (α, β and ε), GATA proteins and cofactors [GATA-1, -2, and FOG (5)], RFX factors and PU.1 on eosinophil target genes (Fig. 2), the majority of which have not yet been defined.

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Regulation of IL5R on eosinophil progenitors in allergic airway inflammation

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Introduction

Recent studies of the involvement of the bone marrow in human atopic asthmatic responses to inhaled allergen confirm what we have found in a canine model of *Ascaris suum*-induced bronchial hyperresponsiveness. $CD34/45^+$ hemopoietic progenitors, increased in numbers in the blood and marrow of atopic individuals, can be specifically upregulated following airway allergen challenge eliciting bronchial hyperresponsiveness and the late-phase response. All the IL-5 responsive subset of progenitors, making the Eo-B lineage specifically, is unregulated in the marrow within 24 h of allergen challenge in dual responder asthmatics. Using triple colour flow cytometry, it can be shown that this subpopulation of progenitors in the marrow is one that bears high affinity receptors for IL-5 (IL-5Rα), existing as a subpopulation of early progenitors bearing CD34/45 (1,2). Thus, the readily mobilizable pool of autocrine [GM-CSF- and IL-5-producing (3)] Eo-B progenitors at a very early stage of lineage commitment is increased after inhalation of allergen, only in those individuals who develop ongoing inflammatory responses. The nature of the signalling between the airway and the bone marrow, which upregulates IL-5Rα on $CD34^+$ progenitors in the bone marrow *in vivo*, is not yet known. We therefore undertook studies to explore the *in vitro* regulation of IL-5R expression on hemopoietic progenitors.

Effects of retinoic acid

IL-5 plays a central role in eosinophil and basophil differentiation, exerting its effects through the IL-5 receptor. Though the α chain of the IL-5R is known to exist as either a membrane-bound or soluble isoform, little is currently known concerning regulation of IL-5Rα gene transcription in the context of commitment of haemopoietic progenitor cells to the eosinophil and basophil lineages.

Recent studies by Tavernier *et al*. have indicated that IL-5 itself can regulate IL-5Rα expression on cord blood-derived mature eosinophils; recent studies in our laboratory indicate that the same holds for bone marrow eosinophil progenitors. Given that all-trans retionic acid (ATRA) is known to modulate some aspects of haemopoietic differentiation, we examined the effects of ATRA on eosinophil/basophil differentiation and IL-5Rα expression. In semi-solid cultures of normal human bone marrow, ATRA selectivity suppressed eosinophil/basophil colony forming units, but had no effect on granulocyte–macrophage colony forming units. Similarly, ATRA inhibited eosinophil/basophil differentiation of cord blood $CD34^+$ cells, while neutrophil differentiation proceeded without impediment. Most importantly, these effects of ATRA or $CD34^+$ cells were associated with selective, dose dependent inhibition of membrane-bound IL-5Rα, upregulation of soluble IL-5Rα transcription, but no change in GM-CSF receptor expression. These findings indicate that retinoids can differentially regulate membrane and soluble isoforms of IL-5Rα, and that these effects have functional consequences *in vitro* on eosinophil and basophil differentiation.

Cord blood studies: prediction of atopy?

The above findings point to an association between allergic asthma and increased responsiveness of myeloid progenitor cells to certain haemopoietic growth factors. However, it is not clear at what age these changes in progenitor cells first becomes manifest, though increasing evidence suggests that the allergic phenotype may begin to emerge in very early life. We therefore compared expression of haemopoietic cytokine receptors on $CD34^+$ progenitor cells in cord blood from normal infants (at 'low risk' for subsequent atopy), and infants with at least one atopic first degree relative ('at risk' for subsequent atopy), by flow cytometry. Although no differences in absolute $CD34^+$ numbers were observed between the two groups, expression of GM-CSF receptor on $CD34^+$ cells was significantly reduced in the 'at risk' compared to the 'low risk' group ($P=0{\cdot}021$), with a tendency to reduced IL-3 and IL-5 receptor expression in the 'at risk' group (4). While the functional sequelae of reduced GM-CSF receptor expression on $CD34^+$ cells remain to be determined, these findings show an association between genetic risk for atopy and changes in the expression of haemopoietic cytokine receptors on cord blood progenitor cells.

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